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Brief Report

Detection of two porcine circovirus type 2 genotypic groups in United States swine herds

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Summary

In late 2005, sporadic cases of an acute onset disease of high mortality were observed in 10- to 16-week-old growing pigs among several swine herds of the United States. Tissues from the affected pigs in Kansas, Iowa, and North Carolina were examined, and porcine circovirus type 2 (PCV2) was detected consistently among these tissues. Phylogenetically, PCV2 can be divided into two major genotypic groups, PCV2-group 1 and PCV2-group 2. Whereas PCV2-group 1 isolates were detected in all the diseased animals, only two of the diseased animals harbored PCV2-group 2 isolates. This observation is important because PCV2-group 1 isolates had never been reported in the United States before (GenBank as of May 16, 2006), and they are closely related to the PCV2-group 1 isolates that have been described in Europe and Asia, previously. Our analysis revealed that each genotypic group contains a distinct stretch of nucleotide or amino acid sequence that may serve as a signature motif for PCV2-group 1 or PCV2-group 2 isolates.

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Porcine circovirus (PCV) is a member of the genus *Circovirus* of the family *Circoviridae* [18, 21]. This family consists of a diverse group of animal viruses that possess a small, closed circular, single-stranded DNA genome that replicate through double-stranded intermediates. PCV has an ambisense circular genome [25] that encodes proteins by the encapsidated viral DNA, and by the minus genome strand of the replication intermediate synthesized in the host. The PCV virion is icosahedral,

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non-enveloped and 17 nm in diameter [25]. Two genotypes of PCV have been identified. PCV type 1 (PCV1) is non-pathogenic, while PCV type 2 (PCV2) has been implicated as the etiological agent of postweaning multisystemic wasting syndrome (PMWS) in swine [1, 5, 11, 23].

The genome nucleotide (nt) sequences of a number of PCV1 and PCV2 isolates have been determined, and their genomes share 68–76% sequence homology [16], whereas PCV1 and PCV2 intra-genotype isolates share 97–99 and 94.6–99% sequence identity, respectively [6, 8]. Two coding regions of opposite polarity (by conventional nomenclature), the replication initiator protein (Rep) on the right and the capsid protein (Cap) on the left, are separated at the 5′-end by the origin of DNA replication (Ori) intergenic region (IR) of approximately 80 nucleotides [4]. The intra-type amino acid homology between the Reps of PCV1 and PCV2 is approximately 99.4 and 95.7%, respectively, while the intra-type amino acid homology between the capsid proteins of PCV1 and PCV2 is 94 and 90%, respectively [8]. Thus, the capsid genes exhibit more sequence divergence than the Rep genes. A third opening reading frame (ORF3) coding for an apoptosis-associated protein has also been reported for PCV2 and it is transcribed in the opposite orientation with respect to Rep [15].

Association of PCV2 with PMWS was first reported in western Canada [5, 11], and then the disease was recognized in Europe [14, 19, 24]. To date, PCV2 has been detected in many parts of the world [22]. In past studies, phylogenetic analyses have shown that PCV2 isolates in various countries can be divided into to two main genotypic groups [6–9, 13, 17]. Recently, an extensive study showed that the two major groups of PCV2 can be further separated into multiple clades: PCV2-group 1 with 3 clades (1A, B and C) and PCV2-group 2 with 5 clades (2A–D and E) [20]. The genome length of group 1 viruses is 1767 nucleotides, while the genome length of group 2 viruses is 1768 nucleotides. However, PCV2 group designations have no apparent association with disease status or geographic area. Interestingly, it has been noted that all of the PCV2 isolates from the United States (USA) until late 2005 belong to group 2 [13, this study] (Fig. 1), while isolates from other countries may be found in both PCV2 groups. In this communication, we report the presence of PCV2-group 1 isolates in multiple USA swine herds.

In late 2005, sporadic cases of an acute onset disease of high mortality were observed in 10- to 16-week-old growing pigs among USA swine herds [2, 10]. Investigations into these cases found the following: there was a sudden increase in mortality that was at least several times the expected weekly rate; there was a high incidence of unthrifty, anorexic, dyspneic and listless pigs; and the onset of this condition was noticed 4–6 weeks post-placement in the finisher. As the epidemic progressed in the barn, more pigs became affected, and by the end of the finishing phase, the mortality rate ranged from 5 to 50% in a given barn of pigs, with case mortality rates reaching 100% when euthanasia and culling for salvage were included. In the months leading up to these epidemics, there were no obvious changes in husbandry practices, feed supply, source of replacement animals, or hygiene practices that would suggest a source of infection or a linkage among the affected herds. At necropsy, the most consistent lesions among the affected pigs observed were enlarged lymph nodes, pneumonia, icterus and variable amounts of pleural and peritoneal effusions. The most consistent microscopic lesions were lymphoid depletion with granulomatous inflammation, nonsuppurative interstitial pneumonia and nephritis.

Frozen tissues from two affected swine herds per state from Kansas, North Carolina and Iowa were submitted to the National Animal Disease Center, Ames, Iowa, for analysis (Table 1). Propagation of various viruses was attempted on multiple cell lines, and cultures that exhibited cytopathic effect (CPE) were then selected for PCR amplification with pathogen-specific primers. Briefly, homogenates (10%) were prepared from various tissues either by trituration with a mortar and pestle, or by using a stomacher. The homogenates were then clarified at $1000 \times g$ for 30 min at 4 °C. The recovered supernatant was passed through a 0.45-micron filter before inoculating onto several established cell lines (MARC-145, PK-15 or ST) and a primary porcine fetal lung cell line grown in 24-well plastic cell

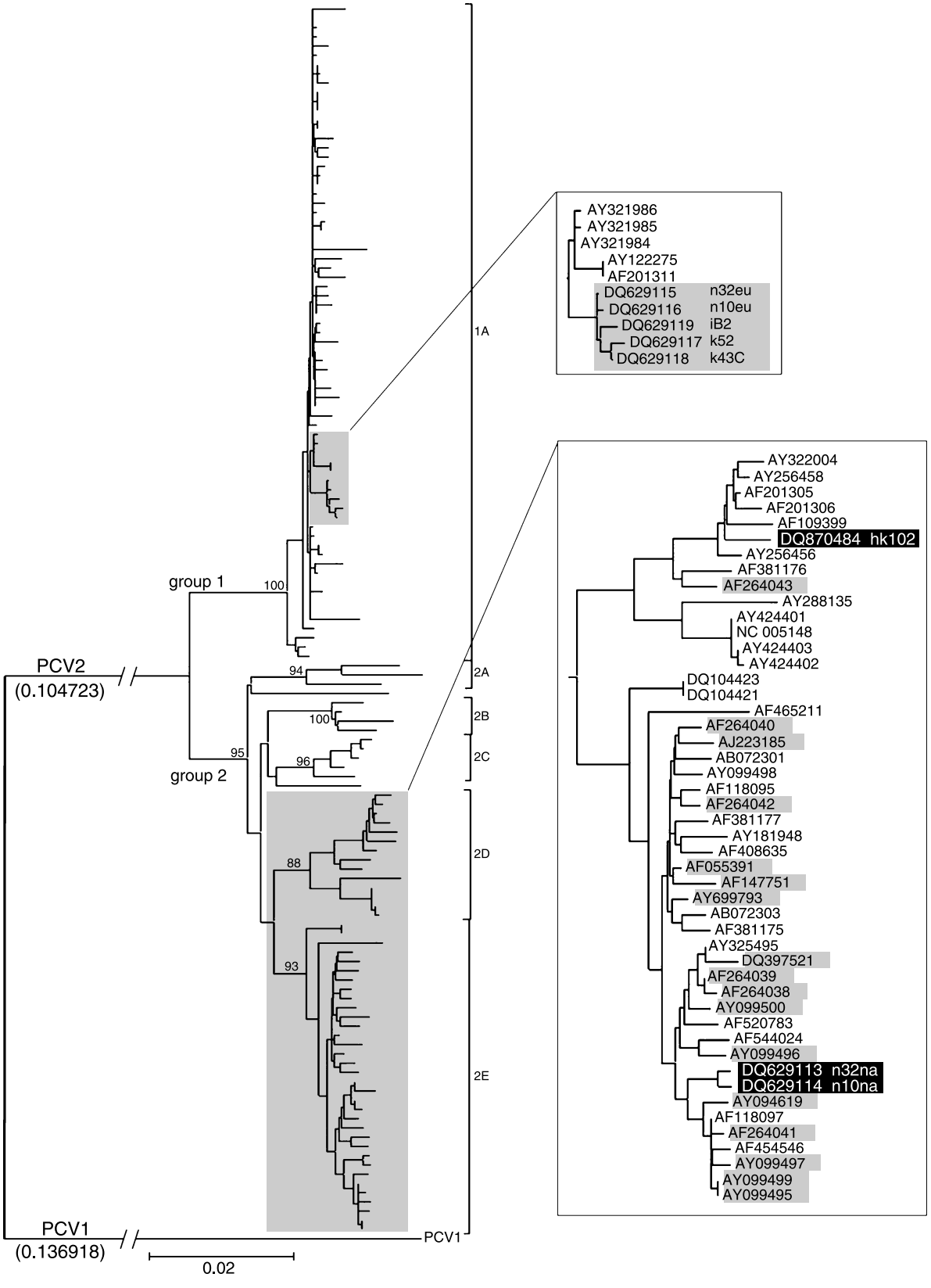
Table 1. Detection of filterable agents by PCR

	Farm	Animal	MARC	ST	PK-15	PFL	PK-15	
							PPV	PCV
North Carolina	1	n3	−	−	PEV	−	−	+
	1	n6	PRRSV	−	−	−	−	+
	1	n10	−	−	−	−	−	+
	1	n13	−	−	−	−	−	+
	1	n19	PRRSV	−	−	−	−	+
	2	n26	PRRSV	PEV	−	−	−	+
	2	n32	PRRSV	PEV	PEV	−	−	+
	2	n40	−	−	−	−	−	+
Iowa	1	iA3	PRRSV	−	−	−	−	+
	2	iB2	−	−	−	−	−	+
	2	iB5	−	−	−	−	−	+
Kansas	1	k43	−	−	−	−	−	+
	1	k47	−	PRCV	−	−	−	+
	1	k52	−	−	−	−	−	+
	1	k58	−	−	−	−	−	+
	2	k63	−	PTV	−	PTV	−	+
	2	k65	−	−	−	−	−	+
	2	k70	−	−	−	−	−	+
	2	k74	−	−	−	−	−	+

Specific CPE-positive cell cultures were analyzed by PCR for *PRRSV* porcine reproductive and respiratory syndrome virus, *PEV* porcine enterovirus, *PRCV* porcine respiratory coronavirus or *PTV* porcine teschovirus. PK-15 cultures incubated with tissue homogenates (with or without CPE) were tested by PCR for porcine parvovirus (PPV) and PCV. Presence or absence of virus in the cell culture is indicated by (+) or (−), respectively. The assays were performed by National Services Veterinary Laboratory, Ames, Iowa. *MARC* MARC-145 cells; *ST* Swine Testicular cells; *PK*-15 Porcine kidney 15 cells and *PFL* Porcine fetal lung cells.

culture plates. The medium was changed 1–2 hours post-inoculation. The culture was maintained in 5% CO_2 atmosphere at 37 °C and observed daily for CPE. All negative cultures were frozen and then inoculated onto 50–75 and 100% confluent fresh tissue culture cells to look for more CPE-positive samples. In addition, PK-15 cells inoculated with tissue homogenates (with or without CPE) were assayed for porcine parvovirus (PPV) and PCV. The results showed that porcine reproductive and respiratory syndrome virus, porcine enterovirus, porcine respiratory coronavirus, porcine teschovirus and PCV, but not PPV, were present in one or more of the diseased animals (Table 1). Since multiple infectious agents were detected in the affected animals, it cannot be assumed that any one agent was responsible for the observed clinical disease.

Nucleotide sequence analysis was carried out to examine the PCV DNAs detected in the diseased animals. Total cell DNA from the tissue homogenates were subjected to PCR with specific primers capable of amplifying both PCV1 and PCV2 DNA [3]. Restriction enzyme and sequence analyses showed that PCV2, but not PCV1, was detected in one or more tissues of all the affected animals examined. For cloning of the complete viral genome, oligonucleotide primers 1010BF (GCATG GATCCATCACTTCGTAATGGT) and 1010BR (GCATGGATCCAAAAAAGACTCAGTAA) were used in PCR amplification, and the PCR products were inserted into a TA-cloning vector for sequence determination. Seven distinct full-length clones (n10eu, n32eu, iB2, k43c, k52, n10na and n32na) from 6 diseased pigs (3 from North Carolina, 2 from Kansas, and 1 from Iowa) were obtained, and the nucleotide sequences have been submitted to GenBank (Table 2). Interestingly, two of the North Carolina pigs (n10 and n32) each contained two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AF201311
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DQ629119 iB2
DQ629117 k52
DQ629118 k43C
AY322004
AY256458
AF201305
AF201306
AF109399
DQ870484 hk102
AY256456
AF381176
AF264043
AY288135
AY424401
NC 005148
AY424403
AY424402
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AF055391
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AB072303
AF381175
AY325495
DQ397521
AF264039
AF264038
AY099500
AF520783
AF544024
AY099496
DQ629113 n32na
DQ629114 n10na
AY094619
AF118097
AF264041
AF454546
AY099497
AY099499
AY099495
group 1
group 2
100
94
100
95
96
88
93
1A
2A
2B
2C
2D
2E
PCV2
(0.104723)
PCV1
(0.136918)
PCV1
0.02

distinct PCV2 sequences, while the sequence from pig n19 was identical to one of the sequences obtained from pig n32. Separately, tissues of a healthy pig (hk102) from a neighboring farm (with no signs of disease) in Kansas were also analyzed, and PCV2 DNA sequences were detected. The eight genomic DNAs obtained here and all the available USA PCV2 sequences (18 entries) reported in GenBank as of May 16, 2006 were subjected to phylogenetic analysis together with the data set of Olvera et al. (which included 8 USA sequences) that was used to delineate the two major genotypic groups of PCV2 [20]. In our analysis, we included one PCV1 sequence (NC006266) and excluded 33 of 148 sequences of the data set: sequences that exhibit recombination characteristics and sequences that were removed for the recombination study (i.e., PCV2-group 1 clade B and clade C sequences and the sequences marked with asterisks, respectively). A total of 134 sequences (Table 2) were aligned by the ClustalW method available in the MEGA 3.1 software (http://www.megasoftware.net) [12]. A phylogenetic tree was constructed by the neighbor-joining method with the Kimura 2-parameter nucleotide substitution model using 1000 bootstrap replicates in the MEGA 3.1 program. The results showed that all the groupings obtained were identical to the results of Olvera et al. [20] (Fig. 1). Clustered within the PCV2-group 2 were the 18 previously reported USA isolates (clades 2D and 2E), the n10na and n32na sequences (clade 2E) (co-isolated with n10eu and n32eu, respectively) from diseased pigs and the hk102 sequence (clade 2D) from the healthy pig from the neighboring farm; while the other five full-length sequences (n10eu, n32eu, iB2, k43c and k52) from six diseased pigs clustered within PCV2-group 1 clade A.

Previous studies had concluded that the PCV2 capsid gene is a suitable phylogenetic and epidemiologic marker [7, 20]. To obtain capsid gene sequences specifically, the DNA samples were amplified with oligonucleotide primers 200R (ATTA CCCTCCTCGCCAAC) and 941F (CCAGTTCGT CACCCTTTC), cloned and sequenced (Table 2). The results showed that the viral DNA sequences from different animals were similar but not identical, and at times, more than one capsid gene sequence was detected in the same animal (n3, n13, k43 and k70). Sixteen new capsid sequences from this study and two additional previously reported USA capsid genes (AY129154 and AY129155) (included in Table 2) were added to the data set for analysis. Together, a total of 152 capsid gene sequences (87 PCV2-group 1, 64 PCV2-group 2 and 1 PCV1) were subjected to phylogenetic analysis by the neighbor-joining method [12]. The results showed that the groupings obtained here were identical to that of the full-length genome analysis (Fig. 2). Clustered within PCV2-group 2 were the 20 previously reported USA isolates (clades 2D and 2E), the n10na and n32na sequences (clade 2E), and the hk102 sequence (clade 2D); while 21 distinct sequences from the diseased animals (not including n10na or n32na) of this study clustered within PCV2-group 1 clade A.

Our analysis revealed several distinct features that are characteristic of either PCV2-group 1 or PCV2-group 2 isolates among the 133 PCV2 complete genomes analyzed (Fig. 3). (1) The PCV2-group 1 sequences (71 entries) are 1767 nucleotides long, while PCV2-group 2 sequences (62 entries) are 1768 nucleotides long. The deleted nucleotide of PCV2-group 1 is located at nt 1040. (2) Rep and ORF3 exhibit fewer nucleotide variations than the capsid gene. The Rep protein has 314 amino acid residues, and 172 out of 942 nucleotide positions (18%) were variable. The most consistent amino acid residue difference is due to a nucleotide change

Fig. 1. Phylogenetic analysis of full-length PCV2 genomes. An unrooted phylogenetic tree was constructed by the neighbor-joining method [12] from aligned sequences: 8 from this study, 18 available USA isolates in GenBank as of May 16, 2006 (distributed within clades 2D and E), 1 PCV1 and 107 PCV2 reference sequences listed in Table 2. The bootstrap value (in percent from 1000 replicates) for each clade is shown. The branch lengths for PCV1 and PCV2 are in parentheses. The scale represents the number of substitutions per nucleotide. The areas of interest are expanded and shown on the right. Upper box: PCV2-group 1 isolates from this study are shaded. Lower box: previous USA isolates are shaded and PCV2-group 2 isolates from this study are in black

Table 2. PCV2 sequences and their GenBank accession numbers

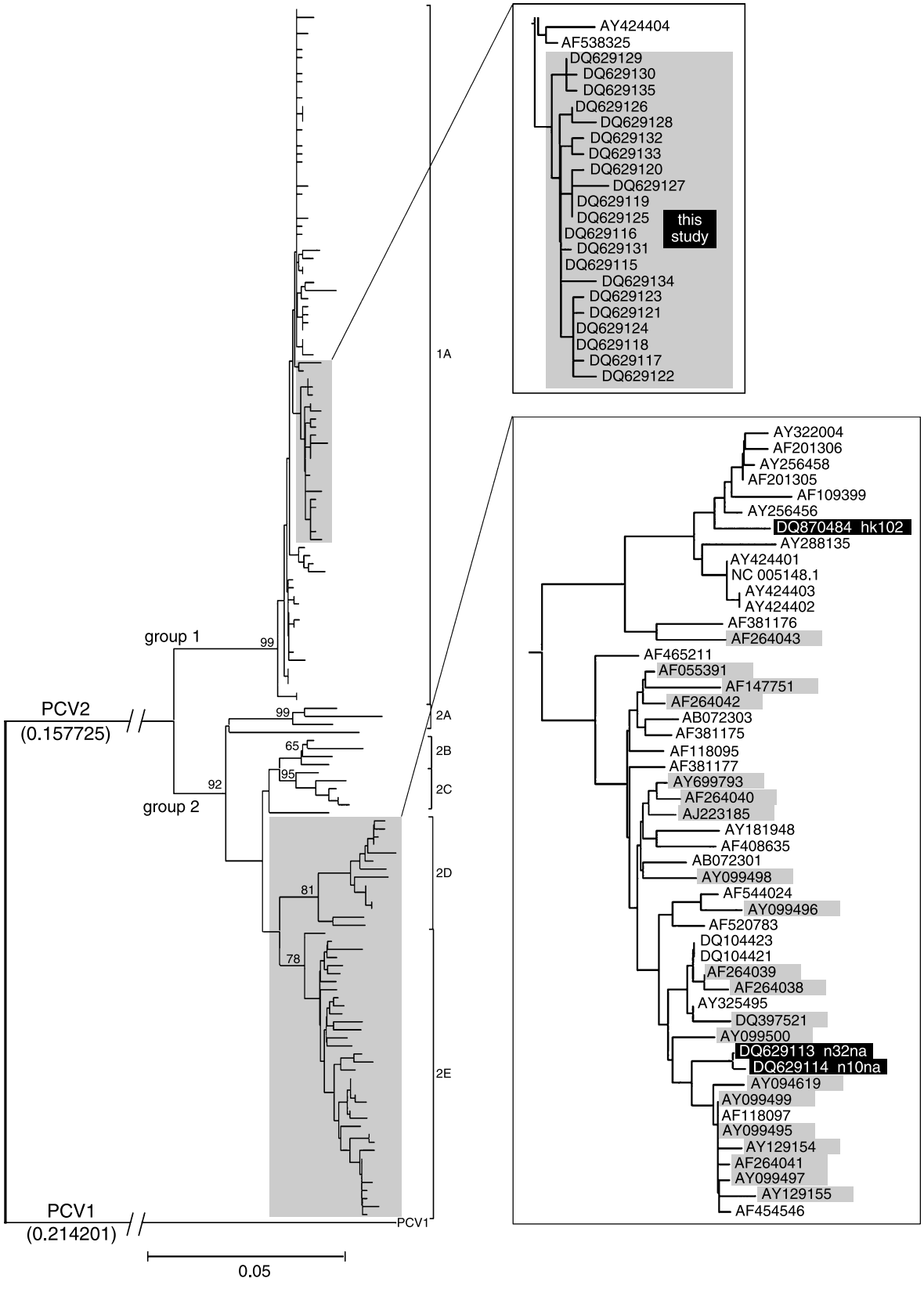
PCV2 in this study	Farm	Animal	Tissue	PCV2 sequence		Accession number
				Genome	Capsid	
North Carolina	1	n3	lung		n3A, n3B	DQ629132, DQ629133
	1	n6	LN		n6C	DQ629134
	1	n10	LN	n10eu*, n10na*		DQ629116*, DQ629114*
	1	n13	LN		n13A, n13B	DQ629129, DQ629130
	1	n19	LN	**n32eu***		DQ629115*
	2	n26	LN		n26A	DQ629131
	2	n32	LN	**n32eu***, n32na*		DQ629115*, DQ629113*
	2	n40	LN		n40A	DQ629135
Iowa	1	iA3	LN		iA3	DQ629120
	2	iB2	LN	iB2*		DQ629119*
	2	iB5	LN		iB5 = **k63F**	DQ629125
Kansas	1	k43	LN	k43C*	k43A, k43B	DQ629121, DQ629122, DQ629118*
	1	k47	LN		k47A	DQ629123
	1	k52	LN	k52*		DQ629117*
	1	k58	kidney		k58A	DQ629124
	2	k63	tonsil		**k63F**	DQ629125
	2	k65	LN		**k65A**	DQ629126
	2	k70	LN		**k65A**, k70B	DQ629126, DQ629127
	2	k74	LN		k74C	DQ629128
	Healthy	hk102	LN	hk102*		DQ870484*

Reference sequence	Accession number
USA PCV2 isolates in GenBank as of May 16, 2006	AY094619, AY099496, AY099497, AY099498, AY099499, AY099495, AY099500, AF2640438, AF2640439, AF264040, AF264041, AF264042, AF264043, AF055391, AF147751, DQ397521, AJ223185, AY699793 (in pepsin), AY129154 (capsid), AY129155 (capsid)
PCV1	NC 006266
PCV2-group 1A and PCV2-group 2 isolates	This list includes 107 PCV2 sequences in Olvera et al. with exclusion of the isolates involved in the recombination study (indicated by asterisks) and isolates that exhibited recombination characteristics (PCV2-group 1B and -group 1C) [20].

The shaded entries are PCV2-group 2 isolates. *LN* Lymph node, * = Full-length genomic clone, and bold letters = identical sequences in two different animals.

located at nt 152, which resulted in an amino acid residue variation with Glu_{62} for PCV2-group 2 and Asp_{68}/Glu_3 for PCV2-group 1 isolates. (The numbers following the amino acid residue indicates the number of times the amino acid residue appeared at this location.) The ORF3-encoded apoptosis-associated protein has 104 amino acid residues, and 51 out of 312 nucleotide positions (16%) were vari-

Fig. 2. Phylogenetic analysis of the capsid gene sequences of PCV2. An unrooted phylogenetic tree was constructed by the neighbor-joining method [12] from aligned sequences: 24 from this study, 20 available USA isolates in GenBank as of May 16, 2006 (distributed within clades 2D and E), 1 PCV1 and 107 PCV2 reference sequences listed in Table 2. The bootstrap value (in percent from 1000 replicates) for each clade is shown. The branch lengths for PCV1 and PCV2 are in parentheses. The scale represents the number of substitutions per nucleotide. The areas of interest are expanded and shown on the right. Upper box: PCV2-group 1 isolates from this study are shaded. Lower box: previous USA isolates are shaded and PCV2-group 2 isolates from this study are in black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AF538325
DQ629129
DQ629130
DQ629135
DQ629126
DQ629128
DQ629132
DQ629133
DQ629120
DQ629127
DQ629119
DQ629125
this study
DQ629116
DQ629131
DQ629115
DQ629134
DQ629123
DQ629121
DQ629124
DQ629118
DQ629117
DQ629122
AY322004
AF201306
AY256458
AF201305
AF109399
AY256456
DQ870484 hk102
AY288135
AY424401
NC 005148.1
AY424403
AY424402
AF381176
AF264043
AF465211
AF055391
AF147751
AF264042
AB072303
AF381175
AF118095
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AF544024
AY099496
AF520783
DQ104423
DQ104421
AF264039
AF264038
AY325495
DQ397521
AY099500
DQ629113 n32na
DQ629114 n10na
AY094619
AY099499
AF118097
AY099495
AY129154
AF264041
AY099497
AY129155
AF454546
group 1
group 2
99
99
65
95
92
81
78
1A
2A
2B
2C
2D
2E
PCV2
(0.157725)
PCV1
(0.214201)
PCV1
0.05

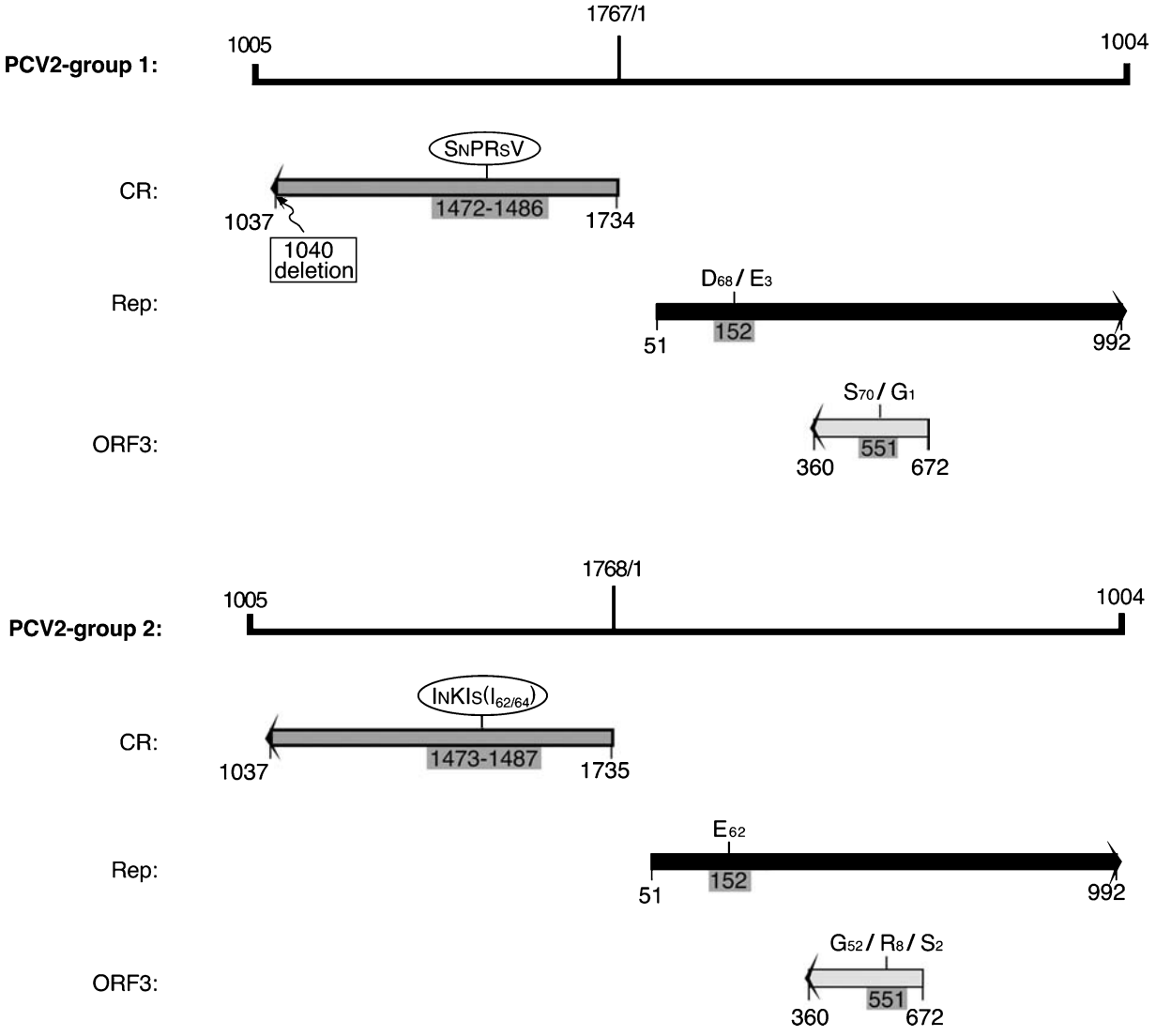

Fig. 3. Comparative analysis of the Rep, ORF3 and Cap genes of PCV2-group 1 and PCV2-group 2 isolates. The Ori of PCV2-group 1 and PCV2-group 2 are indicated by 1767/1 and 1768/1, respectively. Capsid (*CR*) and ORF3 RNAs are transcribed leftward, and Rep is transcribed rightward. The coding regions are annotated with nucleotide coordinates below the gene map. Location of nucleotide differences between PCV2-group 1 and PCV2-group 2 isolates that resulted in amino acid changes (above the gene map) are also indicated. The numbers following the amino acid residue indicates the number of times the residue appeared at this location. The proposed amino acid signature motif is indicated by the circled amino acid sequence above the CR gene map

able. The most consistent amino acid residue difference is due to a nucleotide change located at nt 551, which resulted in an amino acid residue variation with $Gly_{52}/Arg_8/Ser_2$ for PCV2-group 2 and Ser_{70}/Gly_1 for PCV2-group 1 isolates. The capsid protein has 233 amino acid residues, and 201 out of 699 nucleotide positions (28%) were variable. (3) PCV2 isolates can be separated into 2 major genotypic groups based primarily on capsid gene sequences (see Fig. 2). (4) Distinct stretches of nucleotide or amino acid sequences may serve as signature motifs for genotype grouping of PCV2 isolates. The capsid gene nucleotide sequence at position 1486–1472 for PCV2-group 1 (87 entries)

is **T**C**A**/AAC/**CCC**/**CG** (which codes for the 5' portion of amino acid sequence **S**N**PR**S**V**), while the nucleotide sequence at position 1487–1473 for PCV2-group 2 (64 entries) is **A**C**C**/AAC/**AAA**/**AT** (which codes for the 5' portion amino acid sequence **T**N**KI** S[**I**$_{62/64}$]). Whether variation of this cluster of six amino acid residues has any effect on the structure and function of the capsid protein remains to be investigated.

In this communication, we report the presence of PCV2-group 1 and PCV2-group 2 isolates among swine herds in the USA. Whereas only PCV2-group 2 viruses have been reported in the USA prior to this report, both PCV2 groups have been reported in Europe and in Asia [17, 26]. It is not clear how long PCV2-group 1 isolates have been present in swine herds in the USA or the comparative pathogenic capability of these two groups of PCV2. The sudden appearance or increased prevalence of PCV2-group 1 viruses in diseased swine from widely separated locations in the United States suggests that the emergence or import of a new PCV2 genotypic group is a concern for the swine industry.

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